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IBMX-elicited Inhibition of Water Permeability in the Isolated Rabbit Conjunctival Epithelium

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Abstract

Agents expected to increase intracellular cAMP levels were tested on the diffusional water permeability (P_{dw}) of isolated rabbit conjunctival epithelia given recent indications of the apical expression of AQP5, a water channel homologue regulated by cAMP in other cell systems. For these experiments, segments of conjunctivae were mounted between Ussing-type hemichambers under short-circuit conditions. Unidirectional water fluxes (J_{dw}) were measured by adding $^3\text{H}_2\text{O}$ to one hemichamber and sampling from the other, while the electrical parameters (I_{sc} and R_t) were recorded simultaneously. J_{dw} were determined under control conditions and after the introduction of forskolin, dibutyryl-cAMP, rolipram and IBMX. All agents reduced J_{dw} , with rolipram and IBMX the most effective inhibitors ($\approx 28\%$ reduction), while simultaneously evoking stimulations of the I_{sc} ; suggesting that cAMP regulates ionic transport and P_{dw} independently. This observation was consistent with the elimination of the IBMX-elicited I_{sc} stimulations by the PKA inhibitor, H89, and the ineffectiveness of the sulfonamide in preventing the J_{dw} reductions produced by the xanthine. Data from mannitol fluxes and Arrhenius plots indicated that the IBMX-elicited P_{dw} reduction occurred at the level of water-transporting channels, but the specific moiety was not identified. Instead it was observed that lipophiles commonly used in other systems to uncouple cellular communication precluded the effects of IBMX on J_{dw} , but the mechanism for these results was not directly linked to gap-junction blockade in the conjunctiva, as assessed by the transepithelial electrical parameters. Putatively, agents such as heptanol, by also fluidizing the bilayer, may have changed the conformation of a water channel in a manner preventing down-regulation by IBMX. Nevertheless, this study uncovered an apparently unique response to cAMP elevation exhibited by the conjunctiva, namely that P_{dw} declines via an H89-insensitive pathway under conditions whereby PKA-dependent electrolyte transport might be over stimulated due to excessive cAMP levels (e.g., PDE inhibition).

Keywords

unidirectional water fluxes; net water fluxes; Ussing-chamber; short-circuit current; electrolyte transport; cAMP; paracellular mannitol permeability

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INTRODUCTION

Active electrolyte transport by the conjunctival epithelium with accompanying fluid secretion may contribute significantly to the aqueous layer of the tear film. Although this tissue exhibits oppositely directed mechanisms for Na^+ absorption and Cl^- secretion, the latter activity predominates (Shi and Candia, 1995; Candia, 2004), and underpins a spontaneous fluid transport across the isolated epithelium in the stromal-to-mucosal direction (Shiue et al., 2000; Li et al., 2001). The rate of fluid flow is sufficiently large ($\approx 50 \mu\text{l hour}^{-1}$ based upon the total surface area of the conjunctiva) that it may represent a baseline level of tear secretion not attributable to the lacrimal gland (Li et al., 2001). Such transepithelial fluid movement requires not only the net movement of electrolytes across the conjunctival epithelium to the tear-side of the tissue, but also, a highly permeable pathway for water flow, such as that provided by the water channels known as aquaporins (AQPs), as well as, other membrane-spanning proteins including CFTR, some classes of K^+ channels, and the glucose transporter, which all appear capable of contributing to transcellular water movement (Loo et al., 2002; Pohl, 2004).

Generally, epithelia exhibit distinct AQPs in the apical and basolateral domains (King et al., 2004). In the case of the conjunctiva, AQP3 has been localized to the lateral membranes (Verkman, 2003; Oen et al., 2006), and we recently provided evidence for the expression of AQP5 at the apical aspect (Oen et al., 2006), indicating that both corneal and conjunctival epithelia exhibit an identical AQP distribution. Moreover, plasmalemma AQP5 expression levels may be regulated by cAMP-dependent mechanisms (Yang et al., 2003; Sidhaye et al., 2005). Thus, we presently examined if compounds expected to increase the intracellular levels of the cyclic nucleotide exhibited an effect on the diffusional water permeability (P_{dw}) of rabbit conjunctivae.

Hypothetically, the identification of agents serving as up-regulators of conjunctival P_{dw} might be relevant towards future treatments of dry-eye complications in individuals with lacrimal gland dysfunction, by decreasing the barrier to water movement across the conjunctiva. In principle, an increase in P_{dw} could occur in either the apical or basolateral side of the conjunctiva in order to increase water flow across the entire tissue, as we found earlier that neither surface is rate limiting (Candia et al., 1998b). However, our present findings cannot advance this concept, because we determined that acute exposure to cAMP-elevating agents actually decreased conjunctival P_{dw} , with the most pronounced effects obtained with compounds recognized as phosphodiesterase (PDE) inhibitors.

Nevertheless, our present results may not necessarily obviate as potential dry-eye palliatives the use of cAMP-elevating agents, which indeed have stimulatory effects on the Cl^- -secretory activity of the conjunctiva (effects that occur within the same time frame as the down-regulation in P_{dw}), but instead highlight the complex manner by which cAMP may regulate the transport functions of the epithelium.

MATERIALS AND METHODS

Our approach to these studies was to measure the unidirectional flux of tritiated water in the absence and presence of various test compounds. Such unidirectional water fluxes (J_{dw}) across the conjunctival epithelium are statistically identical in either direction (Candia et al., 2006), and have a magnitude ≈ 60 -fold larger than the net flux (J_v) of fluid secreted to the tear side by the isolated conjunctiva ($\approx 4 - 6 \mu\text{l} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$), as reported by Shiue *et al.*, 2000 and Li *et al.*, 2001. Because of this discrepancy in magnitude, it is unfeasible (if not impossible) to calculate J_v as the difference between the two, relatively large, unidirectional fluxes in the opposite directions. Although labeled water will cross cell membranes via all available

pathways – lipid bilayer, AQPs, and other channels, and this flow can be accelerated, or reduced, by affecting any of these pathways, we have demonstrated before that measurements of J_{dw} , which reflect P_{dw} , change equally in both directions when an experimental maneuver changes the water permeability of the epithelium (Candia et al., 1997; 1998a; 1998b; 2006).

The protocols for procuring, isolating and mounting within modified Ussing-type chambers the epithelium of the rabbit conjunctiva were described in detail previously (Shi and Candia, 1995). Briefly, adult albino rabbits of either sex weighing 2–3 kg were rapidly killed by CO₂ asphyxiation, a protocol approved by the Mount Sinai Animal Care and Use Committee. Prior to sacrifice, treatment of the rabbits conformed to our institute's Guiding Principles in the Care and Use of Animals. The bulbar-palpebral conjunctiva was dissected as a cylinder and cut longitudinally to convert it to a flat epithelium that was mounted as a partition between the hemichambers, exposing 0.32 cm² of cross-sectional area. The chambers had the necessary accessories for determinations of transepithelial electrical parameters and vigorous stirring, the latter provided by a continuous bubbling within each hemichamber with a humidified CO₂-air gas mixture. Such rapid bubbling should minimize the influence of unstirred layers during the flux experiments described below, so that unstirred layer effects should be negligible. Because putative unstirred layers are in series with the main membrane and assumed to be constant, only the absolute values in calculated fluxes might be underestimated, but not the observed changes in fluxes elicited by the experimental maneuvers.

Water Fluxes

To measure transepithelial unidirectional water fluxes, J_{dw} (expressed in text as $\mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$), the approach was identical to that used earlier with the amphibian bladder and cornea (Candia et al., 1997; 1998a), as well as in previous work with the rabbit conjunctiva (Candia et al., 1998b; 2006). For this, tritiated water was unilaterally added to one compartment ($\approx 1 \mu\text{Ci ml}^{-1}$ of bathing solution). Thereafter, 2-ml samples from the unlabeled, opposite-side bath were drawn every 15 min, and replaced with fresh bathing solution, thereby maintaining the chamber volume and preventing the “cold-side” activity from exceeding 5% of that of the radiolabeled side. At least four such, “cold-side” samples were obtained for each experimental condition, with a complete experiment usually consisting of three conditions so that paired comparisons could be made. The labeled side was sampled (25 μl) periodically (usually at 30-min intervals) to determine the specific activity and assure its constancy. To prevent a significant reduction of the specific activity from the labeled-side bath during experiments of 3–4 hr duration, the volume of the labeled bath was twice that of the cold side. Samples were mixed with an appropriate fluor and counted in a liquid scintillation detector.

The transconjunctival potential difference was short-circuited (Schoen and Candia, 1978) during the sampling of the ³H₂O fluxes, with the current needed to maintain 0 mVolts across the tissue (i.e., I_{sc}) continuously recorded. Transepithelial electrical resistance (R_t) was determined by measuring (every 5 min) the amount of current necessary to offset the short-circuited condition by ± 3 mVolts for a few seconds.

The bathing medium used during the dissection and control periods of the experiments was a modified Tyrode's solution with the following composition (in mM): 1.8 calcium gluconate, 1.2 MgCl₂, 4.5 KCl, 103 NaCl, 30 NaHCO₃, 1 NaH₂PO₄, 5.7 glucose, 0.3 glutathione and 10 sucrose. The pH of this solution when bubbled with 5% CO₂-95% air was 7.5. It measured ≈ 280 mOsmole kg⁻¹ water. Coils of polyethylene tubing connected to a circulating water bath were placed within each hemichamber to maintain the control temperature of the bathing solutions at 36°C. For the experiments requiring adjustments in medium temperature, which was continuously monitored with a digital thermometer and kept within 0.5°C of the desired temperature, the water in the circulating bath was either heated, or chilled with ice.

Simply for convenience, unidirectional water fluxes were measured in the tear-to-stromal direction in all experiments. Most protocols entailed the sampling (at 15-min intervals) of the control flux over the course of an hour, followed by the apical addition of a test compound with sampling continued uninterrupted for a second hour, after which a second test compound was introduced and sampling continued for a third hour.

Mannitol Fluxes

For these experiments, the 10 mM sucrose of the control Tyrode's solution was replaced by 10 mM unlabeled mannitol, and ^{14}C -D-mannitol was added to the apical-side hemichamber ($\approx 1 \mu\text{Ci ml}^{-1}$ of bathing solution) at $t=0$ of the flux protocol. Samples (2 ml) were then taken from the unlabeled bath and replaced with fresh solution at 15 min intervals for 2 experimental conditions comprising a 2-hr duration. During the course of the experiment, the labeled side was sampled (25 μl) periodically to calculate the specific activity. All samples were mixed with a modified Bray's solution and the beta emission was counted in a liquid scintillation detector.

Definition of Coefficients

The diffusion permeability coefficient, P_{dw} , is related to J_{dw} by the following equation:

$$P_{dw} = J_{dw} / (A \cdot V_w \cdot C_w)$$

Where A is the area of the membrane (cm^2), V_w is the partial volume of water ($\text{cm}^3 \text{mol}^{-1}$), C_w is the concentration of water (mol cm^{-3}), and J_{dw} is the measured flux in $\text{cm}^3 \text{s}^{-1}$. In our analysis, J_{dw} has the meaning of a unidirectional flux that can cross the membrane by any available pathway. This equation was also used to calculate changes in P_{dw} as a function of bath temperature for the construction of Arrhenius plots.

Data Analysis

The significance of experimentally elicited changes in water diffusion rates were analyzed using Student's t -test as paired data, with $\alpha = 0.05$ chosen as the level of confidence.

Chemicals

Tritiated water was purchased from ICN Biochemicals, Costa Mesa, CA, USA. Calbiochem (La Jolla, CA) was the source of the isoquinolinesulfonamide, H89, which was stored at 5°C in aqueous solution (10 mM). All other chemicals, including D-mannitol- ^{14}C , were obtained from the Sigma-Aldrich Company, St. Louis, USA. Stocks (100- to 1000-fold solutions) of all test compounds were prepared in either water, ethanol, or dimethyl sulfoxide based upon solubility data provided by Sigma-Aldrich. All such solutions were refrigerated and consumed within 2–3 months, save dibutyl-cAMP (db-cAMP), which was freshly prepared immediately prior to use.

RESULTS

Effects of pharmacological augmentation of cAMP levels on J_{dw} and electrical parameters

Earlier characterizations of isolated rabbit conjunctival epithelia in a bicameral Ussing-type arrangement demonstrated that cAMP-elevating agents stimulate the transepithelial I_{sc} as a consequence of simultaneously increasing apical Na^+ and Cl^- , and basolateral K^+ conductances (Turner et al., 2000). Given recent indications in lung cells that cAMP affects the distribution of AQP5 (Yang et al., 2003; Sidhaye et al., 2005), a water channel found at the apical aspect of the conjunctival epithelium (Oen et al., 2006), our present work determined the effects of experimental maneuvers expected to increase the intracellular levels of cAMP on the diffusional water permeability of the conjunctiva.

For these studies, J_{dw} were determined under control conditions and after the introduction of either a lipid permeable cAMP analogue, db-cAMP; an artificial adenylyl cyclase activator, forskolin (FSK); and the phosphodiesterase (PDE) inhibitors, IBMX and rolipram (selective for cAMP-PDE IV) (Reeves et al., 1987). All agents reduced J_{dw} , with rolipram and IBMX the most effective inhibitors tested (Table 1). These latter compounds (100 μ M) respectively lowered J_{dw} from 7.91 ± 1.21 to $5.56 \pm 0.87 \mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$ ($n=8$, $P<0.001$, as paired data; Table 1 values from Protocol D), and from 7.96 ± 1.15 to $5.76 \pm 0.80 \mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$ ($n=14$, $P<0.001$, as paired data; combined values from Table 1, Protocols B & E); representing reductions in water permeability of $\approx 28\%$. In contrast, the inhibition in J_{dw} elicited upon db-cAMP addition (1 mM) was limited to an $\approx 8\%$ change, from 7.56 ± 1.16 to $6.92 \pm 0.98 \mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$ ($n=8$, $P<0.02$, as paired data; Table 1, Protocol A). Similarly, with preparations administered FSK as the initial test compound (Table 1, Protocol C), a relatively limited 4% reduction in J_{dw} was obtained, from 6.22 ± 0.80 to $5.96 \pm 0.81 \mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$ ($n=10$, $P<0.05$, as paired data). However, the sequential introduction of IBMX to preparations initially exposed to db-cAMP and FSK (Table 1, Protocols A & C) evoked additional inhibitions in J_{dw} of 14 % and 25 %, respectively, suggesting that inhibition of PDE was necessary to elicit salient effects on the water fluxes.

A successive, 12% inhibition in J_{dw} was also obtained upon introducing IBMX to rolipram-pretreated tissue (Table 1, Protocol D), but the administration of these drugs in reverse order (Table 1, Protocol E) did not result in a further significant decline in J_{dw} when rolipram was added after the xanthine, implying that IBMX was the most effective inhibitor of the water fluxes in the conjunctiva. Although it is possible that the effects of the xanthine may be contributed in part by the capacity of IBMX to affect other activities beyond its inhibition of PDE, e.g., IBMX is a known antagonist of P1 type purinergic receptors, etc., such determination of any additional biochemical effects of IBMX is beyond the scope of the present survey given that rolipram (a PDE IV inhibitor) was also a very effective J_{dw} inhibitor.

All of the putative cAMP-elevating agents that were tested evoked their established, virtually instantaneous stimulations of the I_{sc} (Table 1), suggesting that cAMP may regulate ionic transport and water permeability independently. As observed earlier (Turner et al., 2000), additive effects by the cAMP-elevating drugs on the I_{sc} only occurred when the agents were successively applied in certain combinations, e.g., the addition of IBMX to conjunctiva pretreated with db-cAMP (Table 1, Protocol A), or the addition of epinephrine to IBMX-pretreated conjunctiva (Turner et al., 2000). In contrast, the combinations of IBMX and FSK, and of IBMX and rolipram (irrespective of the order of addition) did not result in sequential, additive I_{sc} stimulations, but rather, an I_{sc} decline upon introducing the second compound (Table 1, Protocols B through E). The mechanism underlying the I_{sc} inhibition produced upon addition of the second drug has not been determined, but it appears likely that a negative-feedback process is involved. Nevertheless, it was clear that the initial administration of a cAMP-elevating agent produced a decline in J_{dw} with a concomitant I_{sc} stimulation, whilst the addition of a second cAMP-elevating drug generally evoked a further J_{dw} decline and an I_{sc} inhibition (Table 1).

All of the cAMP-elevating agents, when applied as the initial test compound (Table 1), produced a reduction in R_t as a consequence of activating conjunctival ionic conductances (Turner et al., 2000). Additional R_t changes were usually not observed upon introducing a second cAMP-elevating drug (Table 1).

Transconjunctival water fluxes seem to be predominately transcellular based on several observations that we detailed before (Candia et al., 2006), with the most significant being the independence between J_{dw} and the integrity of the paracellular pathway. As such, subsequent

water-flux tables presented below show only J_{dw} and I_{sc} values, with the latter given to corroborate that a given agent evoked its expected electrical effect on the tissue.

Arrhenius plots from temperature-dependent measurements of P_{dw} in the absence and presence of IBMX

Water fluxes were measured across conjunctival epithelia at three temperatures (286°K, 299°K, and 312°K) under control conditions; thereafter, IBMX was added to the tear-side hemichamber and J_{dw} measurements were repeated in the same preparations at the same 3 temperatures used to obtain the control values. In this manner, paired data was acquired to construct Arrhenius plots for the control and xanthine-treated conditions. The slopes of the plots of $\ln P_{dw}$ versus $1/^\circ\text{K}$ represent the activation energy (E_a) for water movement across the epithelium; a comparatively greater slope depicting a relatively higher E_a level implies that proportionally more transepithelial diffusion is occurring across the lipid bilayer, that is, there is less water movement through water channels (Verkman et al., 1996).

Four individual control plots and 4 experimental plots were constructed from measurements with 4 conjunctivae. On average, the control diffusional water permeabilities (P_{dw}) were 0.59×10^{-4} , 0.88×10^{-4} , and 1.29×10^{-4} cm/sec at 286°K, 299°K, and 312°K, respectively, while the control E_a over the range of the 3 temperatures was 5.35 ± 0.44 kcal/mole, a level consistent with water movement predominately through aqueous pores (Verkman, 1992; Verkman et al., 1996; Zhang and Verkman, 1991). In the presence of IBMX, the slope increased by 30% to 6.97 ± 0.61 kcal/mole (means $\pm$ SE's, $n = 4$; $P < 0.02$, as paired data). The composite results (plotted in Fig. 1 with the linear equations for the 2 conditions) suggest overall that IBMX treatment probably led to an inhibition of water-transporting channels.

Absence of possible role of PKA in observed IBMX-elicited J_{dw} inhibition

The compound H89, a member of the H-series of chemically synthesized kinase inhibitors of Hidaka and coworkers (Hidaka et al., 1991; Hidaka and Kobayashi, 1992) with the highest affinity for PKA, was tested on J_{dw} (Table 2). Its addition (10 μM) to the apical-side bathing solution led to an immediate $\approx 40\%$ I_{sc} decrease (Table 2, Protocol A) without significantly affecting J_{dw} . The subsequent introduction of IBMX did not markedly alter the I_{sc} , consistent with the importance of PKA to transconjunctival I_{sc} stimulations that are elicited upon PDE inhibition (Turner et al., 2000), but did produce an $\approx 28\%$ reduction in J_{dw} , a change similar to that observed under control conditions (Table 1, Protocols B & E). Because of indications that H89 has a limited permeability in cell membranes (Hidaka et al., 1991; Hidaka and Kobayashi, 1992), a second protocol tested the effects of IBMX on J_{dw} in conjunctiva pre-exposed to the sulfonamide for 2h prior to the introduction of tritiated water and the initiation of the sampling from the unlabeled side of the epithelium (Table 2, Protocol B). Even in this case, with which the epithelium had been bathed by H89 for a total of 3h at the time of IBMX addition, the xanthine also produced a J_{dw} inhibition ($\approx 28\%$). A total inhibition of PKA activity by this second protocol is suggested by the complete elimination of the stimulatory effects of IBMX on the I_{sc} , which requires cAMP/PKA-dependent modulation of CFTR and K^+ channels (Turner et al., 2000). Moreover, because H89 can potentially inhibit additional kinases (Davies et al., 2000; Lochner and Moolman, 2006), clearly none of these other putative enzymes, whose activities may have also been impaired, are involved in the IBMX-evoked inhibition of J_{dw} .

Absence of possible role of the cytoskeleton in observed IBMX-elicited J_{dw} inhibition

Given indications that the microfilament network is essential for the retrieval of water channels from the apical membrane (Dibas et al., 2000), separate sets of experiments examined the effects of cytochalasin B, a microfilament-disrupting reagent, and for comparative purposes, the effects of the microtubule disruptor, colchicine, on conjunctival J_{dw} (Table 3). Both compounds (30 μM) inhibited control levels of J_{dw} significantly (by $\approx 20\%$ and 7%,

respectively), but only the former reduced the I_{sc} and prevented a current stimulation by IBMX, which instead produced an I_{sc} decline (Table 3, Protocol A). Moreover, the inhibitory properties of the xanthine on J_{dw} were not precluded by the presence of these cytoskeletal reagents, with respective inhibitions of 19% (Table 3, Protocol A) and 28% (Table 3, Protocol B). In both protocols, the combined presence of IBMX and the cytoskeletal disruptor lowered J_{dw} to levels ≈ 33 –35% below that of the control flux.

Overall, these observations with the conjunctiva indicate that the effects of IBMX on J_{dw} cannot be attributed to an H89-sensitive endocytosis of surface membrane water channels, as reported in the case of AQP5 in murine lung epithelial cells, with which this aquaporin is removed from the membrane as part of an acute response to elevated cAMP levels; a translocation prevented by H89 treatment (Sidhaye et al., 2005).

Effects of IBMX on J_{dw} in amphotericin B-permeabilized conjunctival preparations

The ionophore amphotericin B has been used as an artificial water channel in lipid bilayers (Holz and Finkelstein, 1970), and to increase the water permeability of the apical surface of the frog corneal epithelium (Candia and Zamudio, 1995; Candia et al., 1998a). With the conjunctiva, this polyene antibiotic does not have marked stimulatory effects on J_{dw} ; instead there is a tendency for J_{dw} to decline by a few percent in its presence (see Table 5 in Candia et al., 1998b), a finding that we also obtained presently; a 6% reduction from 8.24 ± 0.80 to $7.74 \pm 0.81 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ ($n = 4$, $P < 0.05$, as paired data). The reason for this decrease is not immediately apparent [perhaps ion channels capable of mediating water movement closed under the extensive depolarization of the membrane potential caused by the ionophore (Candia and Cook, 1986)], but because the present results markedly contract the 20% stimulation of J_{dw} with amphotericin B in the frog cornea (Candia et al., 1998b), the results with the rabbit conjunctiva suggest that the intrinsic water permeability of the apical face of the conjunctiva is sufficiently high that the introduction of additional artificial water channels from the insertion of the ionophore in the bilayer does not increase the transconjunctival water diffusion; i.e., the apical surface is not rate limiting to J_{dw} . In the presence of amphotericin B, the subsequent introduction of IBMX further reduced J_{dw} by 21% to $6.12 \pm 0.44 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ ($n = 4$, $P < 0.05$, as paired data). This latter effect suggests that the IBMX-elicited reductions in J_{dw} may be independent of apical AQP5 activity, because it is highly likely that amphotericin B shunted the native aquaporins present. These results imply that the target of the IBMX-evoked down-regulation in J_{dw} is not in the apical membrane.

Effects of IBMX on J_{dw} in the presence of various channel blockers

In a series of 5 sets of experiments (Table 4), the addition of the xanthine consistently reduced J_{dw} across conjunctivae pretreated with compounds expected to either inhibit conjunctival Cl^- and/or K^+ conductances (Alvarez et al., 2005; Turner et al., 2000), or possibly block AQP3. For the latter, the effects of CuSO_4 (1 mM) were screened based on a report that Cu^{2+} reduces water permeability in cells expressing AQP3 (Zelenina et al., 2004). We found that its bilateral addition reduced J_{dw} by 14% (from 5.29 ± 0.48 to $4.56 \pm 0.44 \mu\text{l} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ ($n = 15$, $P < 0.001$, as paired data). In 4 of these experiments (Table 4, Protocol A), IBMX was introduced following Cu^{2+} , eliciting a 19% reduction in J_{dw} beyond that produced by Cu^{2+} . Moreover, the addition of Cu^{2+} led to a gradual halving of the I_{sc} and R_t , and prevented an I_{sc} stimulation upon IBMX introduction (Table 4, Protocol A), an observation suggestive of a metabolic effect by Cu^{2+} on cellular function in addition to any putative AQP3 blockade.

Glibenclamide, which is recognized as a blocker of Cl^- conductances mediated by CFTR, as well as a variety of other Cl^- and K^+ channels, and affects numerous intracellular enzymes including PKA (Schultz et al., 1999), inhibits conjunctival Cl^- and K^+ conductances (Alvarez

et al., 2005) and J_{dw} (by 11%; Table 4, Protocol B). However, its presence did not prevent a 14% J_{dw} reduction and 37% I_{sc} stimulation upon IBMX addition (Table 4).

Likewise, the application of NPPB, which blocks various Cl^- (Keeling et al., 1991; Schultheiss and Diener, 1998), and nonselective cation channels (Popp et al., 1993), also evoked a reduction in J_{dw} (9%) without preventing a further 13% decline in the water fluxes by IBMX (Table 4, Protocol C). NPPB did, however, prevent an I_{sc} stimulation by the xanthine.

Niflumic acid, also a fenamate modulator of a variety of Cl^- and K^+ channels (Wang et al., 1997; Wu, 2003; Greenwood and Leblanc, 2007), as well as intracellular enzymes, such as COX2, etc., was the only potent conjunctival I_{sc} inhibitor that we screened, which was ineffective on J_{dw} (Table 4, Protocol D). Conjunctivae pretreated with this drug exhibited a robust 115% I_{sc} stimulation and 15% J_{dw} decline upon IBMX addition.

The effects of the relatively nonselective K^+ blocker, Ba^{2+} , were also determined. Its application elicited a 12% J_{dw} reduction and 76% elimination of the I_{sc} (Table 4, Protocol E). The sequential addition of IBMX produced a further 18% decline in J_{dw} without affecting the I_{sc} ; the latter effect expected due to the elimination of the driving force for conjunctival Cl^- secretion and Na^+ absorption by the presence of Ba^{2+} (Turner et al., 2000).

Overall, although there are subtypes of Cl^- and K^+ channels with reported water permeabilities as large as aquaporins (see Fig. 4 in Pohl, 2004), and this screening of relatively nonselective channel blockers evinced agents capable of reducing conjunctival J_{dw} , none precluded a sequential IBMX-evoked J_{dw} inhibition, suggesting that IBMX is affecting a target unaffected by the channel blockers that were tested.

Effects of gap junction blockers on J_{dw} and the IBMX response

To examine the relative contribution of gap junctions to transepithelial water movement, a series of experiments tested the effects of agents reported to uncouple cell communication in various systems (Rozental et al., 2001; Spray et al., 2002) under control conditions (Table 5). These results are presented with the caveats that 1) there are no potent and selective gap junction blockers (Rozental et al., 2001), 2) all of the agents identified as such may cause inhibition of gap junctional conductance via different mechanisms (Rozental et al., 2001; Spray et al., 2002), and 3) each of the drugs that we used have known effects on various other cellular processes including direct activation or inhibition of ion channels (Tewari et al., 2000; Marcet et al., 2004). However, each of these agents precluded a sequential, salient effect on J_{dw} by IBMX, although each uncoupling agent exhibited distinct effects, in themselves, on control levels of J_{dw} and the I_{sc} (Table 5). The only common property shared by the tested uncouplers that we are aware of is that these compounds are all lipophiles capable of increasing membrane fluidity (Goldstein, 1984; Rozental et al., 2001).

Octanol applied at 2mM (Table 5, Protocol A) transiently (2–3 min) stimulated the I_{sc} prior to its gradual elimination and did not affect J_{dw} . The presence of the alkanol also prevented subsequent electrical effects by IBMX and limited the effect of the xanthine on J_{dw} to a $\approx$ 5% decline ($n=7$; $P<0.05$, as paired data), which was the only significant inhibition obtained upon pre-exposure to the uncoupling agents that were tested (Table 5). The initial I_{sc} stimulation produced by octanol may be related to a direct activation of CFTR (Marcet et al., 2004) prior to succeeding gap junction closure.

Heptanol was tested at 5 and 10 mM concentrations (Table 5, Protocols B & C). The latter concentration was needed to evoke an I_{sc} decline as large as that obtained with 2 mM octanol (Protocol A), while the former concentration reduced the control I_{sc} by about 50% (Protocol B); this current change is similar to that reported earlier with 4.5 mM heptanol (Shi and Candia,

1995). More importantly, neither concentration of heptanol affected J_{dw} significantly, whilst both prevented subsequent effects on the water fluxes by IBMX addition, which did not evoke a significant effect on the I_{sc} (Table 5).

Treatment with 1mM oleamide (Table 5, Protocol D) reduced J_{dw} by 7% ($n=6$; $P<0.05$, as paired data) and simultaneously elicited a rapid and stable 32% I_{sc} rise ($P<0.02$, as paired data), which was further augmented (by 62%) to $30.2 \mu A \cdot cm^{-2}$ upon IBMX addition. These IBMX-evoked I_{sc} stimulations in the presence of oleamide persisted as an elevated plateau above the baseline-current levels throughout the duration of the experiments. But no effect by IBMX on J_{dw} was obtained.

I_{sc} stimulations (69%) were also obtained with 200 μM arachidonic acid (Na^+ salt) beyond which IBMX produced an additional 10% I_{sc} increase ($n=5$; $P<0.05$, as paired data) without any significant change in the water fluxes under these conditions Table 5, Protocol E).

Overall, in these sets of experiments (Table 5), only oleamide inhibited J_{dw} , and an IBMX-evoked reduction in the water fluxes only occurred in the presence of 2 mM octanol, an inhibition (7%) that was markedly less than the 28% reduction obtained under control conditions (Table 1). And, while these agents all attenuated an effect by IBMX on J_{dw} , oleamide and arachidonate prevented the IBMX effect and stimulated the I_{sc} at relatively low concentrations. The current stimulations by these latter lipophiles are likely related to the activation of epithelial Cl^- conductances mediated by $ClC-2$ channels (Tewari et al., 2000), which are expressed in the conjunctiva (Turner and Wolosin, 2004).

Effects of IBMX on mannitol fluxes and R_t

Given reports that cAMP-elevating agents could increase the barrier properties of some cellular systems (Duffey et al., 1981; Stelzner et al., 1989), a set of experiments was conducted to determine if IBMX affected transconjunctival mannitol fluxes, which are routinely used as a marker for paracellular permeability because it is well established that mannitol does not cross cell membranes. In these experiments, 7 conjunctivae coincidentally exhibiting control electrical resistances on the lower end of the range of R_t s that can be obtained ($0.48 \pm 0.07 k\Omega \cdot cm^2$) were used. However, the control mannitol permeability ($1.66 \pm 0.27 \times 10^{-6} cm/sec$) was similar in magnitude to that obtained earlier with preparations expressing higher R_t s (Candia et al., 1998b; 2006), suggesting that the present set of data represented epithelia with relatively larger baseline electrical conductances. The addition of IBMX to these preparations increased the I_{sc} by 65% from 17.4 ± 1.9 to $28.6 \pm 5.3 \mu A \cdot cm^{-2}$ ($n=7$; $P<0.05$, as paired data), a change concomitant with an 17% R_t decline (to $0.40 \pm 0.04 k\Omega cm^2$), but one that occurred without an effect on the flux of mannitol (0.060 ± 0.010 vs. $0.062 \pm 0.010 \mu mole \cdot h^{-1} \cdot cm^{-2}$ ($n=7$, $P>0.4$, as paired data). These results indicate that IBMX solely influences transcellular pathways across the conjunctiva, at least within the time frame of the present experiments.

DISCUSSION

Our work evinced a feature apparently unique to the conjunctival epithelium, namely that cAMP stimulates the I_{sc} via PKA activity while simultaneously down-regulating P_{dw} by an H89-independent pathway that remains undefined. These findings were unexpected and have not been described.

The application of cAMP-elevating compounds known to rapidly increase the transconjunctival I_{sc} also reduced J_{dw} (Table 1) as a consequence of a P_{dw} decrease due to the likely inhibition of “water-transporting” channels (Fig. 1). Such channels include the aquaporins, AQP3 and AQP5, which are expressed by the conjunctiva (Oen et al., 2006); ionic channels capable of mediating significant water movement across biomembranes (Pohl,

2004); and/or hemichannels, which also have the capacity to transport water (Orce et al., 2004) and comprise the communicating junctions within the multilayered epithelium along the stratification axis. We do not yet have potent and selective pharmacological blockers that might enable an elucidation among these possible pathways.

Mercurial reagents, for example, have been used to inhibit water flow via Hg^{2+} -sensitive aquaporins (Kuwahara et al., 1997), which include those found in the conjunctiva. However, although the introduction of 0.5 mM HgCl_2 to the tear-side bath of isolated conjunctivae under short-circuited conditions reduced J_{dw} by $\approx 6\%$, the mercurial also rapidly eliminated the I_{sc} within 6–8 min and irreversibly compromised R_t bringing the latter parameter to levels near that of the solution resistance (Candia et al., 2006); overall suggesting that the well-documented cytotoxic effects of Hg^{2+} (Issa et al., 2003; Kim and Sharma, 2004; Shih et al., 2005) might have come into play and adversely affected the integrity of the epithelium. This caveat must also be considered when judging the effects of Cu^{2+} and Ni^{2+} , which are touted as potential blockers of water transport via AQP3 (Zelenina et al., 2003; 2004). The soluble forms of these metal cations can readily enter the epithelium and replace the metal ions that are generally required for the structure and function of many enzymes that use metal ions as prosthetic groups, thereby leading to their disruption, or inactivation (given the relatively high bath concentrations of these divalents that are needed to reduce J_{dw}) (Alt et al., 1990; Ke et al., 2006; Kishimoto et al., 1992; Valko et al., 2005). Yet, in the presence of 1 mM Cu^{2+} , which reduced the I_{sc} and R_t markedly, an IBMX-evoked reduction in J_{dw} persisted without a stimulation of the I_{sc} (Table 4), implying that the specific biochemical pathway(s) responsive to the mediation of J_{dw} by the xanthine was not affected by the metal, and perhaps, the J_{dw} reduction may not involve AQP3.

Furthermore, the apparent down-regulation of J_{dw} by IBMX was not consistent with a possible endocytosis of AQP5 via an H89-sensitive mechanism, as found with lung epithelial cells, with which such retrieval occurs as part of the acute response to elevated cAMP levels (Sidhaye et al., 2005). The independence of the conjunctival J_{dw} response to IBMX exposure from H89 pretreatment (Table 2), and from the depolymerization of the cytoskeleton (Table 3), precludes a possible similarity to the above-mentioned short-term effects of cAMP on AQP5 translocation. Moreover, the persistence of an IBMX-evoked reduction in J_{dw} in the presence of amphotericin B, which conceptually shunts the endogenous AQP5 channels resident in the tear-side surface membranes (Oen et al., 2006), is suggestive that the target of the IBMX effect does not reside within the apical domain.

None of the relatively nonselective ion channel blockers that were screened (Table 4; Protocols B-E) precluded a subsequent effect by IBMX on J_{dw} , although most elicited a reduction in J_{dw} and all caused substantial I_{sc} inhibitions. If ion channels are involved in the IBMX-elicited J_{dw} reduction, we have been unable to identify such channels.

Moreover, the relative effectiveness of IBMX and rolipram as J_{dw} inhibitors, in contrast to that of forskolin and db-cAMP (Table 1), leaves us with having to speculate that the explanation for these observations resides in the complexity of cell signaling via cAMP, which is now increasingly recognized as highly compartmentalized. Recent work has discovered a score of PKA-anchoring proteins (Barnes et al., 2005; McSorley et al., 2006; Smith and Scott, 2006; Zaccolo, 2006) serving as molecular scaffolds tethering the kinase in close proximity to adenylyl cyclase, PDE IV, and a target of cAMP signaling, such as CFTR (Barnes et al., 2005). In this manner, cAMP is confined within a sub-cellular space because PDE IV serves as a barrier that prevents the nucleotide from diffusing throughout the cytosol (Barnes et al., 2005). PDEs are the only known cAMP-degrading enzymes and their incorporation in the PKA-anchoring protein complex results in cAMP gradients that regulate PKA locally (Zaccolo, 2006).

Such compartmentalization, which may be ubiquitous (Smith and Scott, 2006), implies that disruption of the cAMP-diffusion barrier, by treatments with rolipram and IBMX led to marked elevation of cAMP throughout the cell compartment, so that PKA-independent, cAMP effectors could have come into play upon PDE inhibition; thereby enabling the second messenger to produce the J_{dw} reduction. This possibility would explain the potency of forskolin on I_{sc} stimulations and its relative ineffectiveness in reducing J_{dw} , since the PDE IV barrier would have remained unperturbed. In contrast, the lipid-soluble analogue, dibutyryl-cAMP, may bypass the barrier due to its relatively low affinity for PDE IV (Swislocki, 1970), thereby rendering the compound relatively more effective than FSK (Table 1).

Given that the most pronounced J_{dw} inhibitions occurred in the presence of PDE inhibitors (Table 1) and such inhibitions did not require PKA activity, as judged by the results with H89 (Table 2), it would appear that the cAMP effector that produced the J_{dw} reduction required relatively high levels of cAMP, i.e., the unidentified effector exhibits a lower affinity for cAMP than does PKA. A possible candidate for such an effector could be one of the recently discovered cAMP-binding proteins that in turn up-regulate Ras-like small GTPases (Bos, 2006; Holz et al., 2006). Such cAMP-binding proteins (also known as exchange proteins activated directly by cAMP, or Epac's) have now been implicated in multiple cellular functions, e.g., integrin-mediated cell adhesion, cell barrier formation, membrane protein translocation, etc., with many other processes expected to be identified (Holz et al., 2006). Thus, it is conceivable that such a novel signaling transduction mechanism could modulate the activity of a water-transporting channel in the conjunctiva.

Independently of the putative pathway by which cAMP might elicit its regulatory effects on P_{dw} , the cellular element affected by the IBMX exposure remains to be identified. Initially, we considered two possibilities that have been described in other systems: 1) direct binding of cAMP to connexin-32 and -26 that result in channel closure with relatively high millimolar levels of the cyclic nucleotide (Bevans and Harris, 1999); and 2) an increase of the membrane fluidity of human erythrocytes exposed to 10^{-6} to 10^{-5} M levels of db-cAMP (Tsuda et al., 1999).

Neither possibility appears especially satisfying as an explanation for the J_{dw} reduction evoked by IBMX. Firstly, the conjunctiva primarily expresses connexins 31 and 43 (Turner and Wolosin, 2004), and there is presently no evidence that these isoforms can directly bind cAMP. Secondly, a putative direct effect by cAMP on membrane fluidity might be expected to increase J_{dw} across the more disordered bilayer (Brasitus et al., 1986; Giocondi and LeGrimellec, 1991; Ives and Verkman, 1985; Kutchai et al., 1980; Lande et al., 1995; Worman et al., 1986). The only manner that we can envision an increase in fluidity to result in reduced water fluxes is to have the conformation of a water-transporting channel altered to a closed position, as modeled for the spatial change in connexin arrangement that results in gap-junction closure in fluidized membranes (e.g., see Fig. 1 in Lerner, 1997).

The lipophiles that we used to prevent an IBMX-elicited J_{dw} inhibition (Table 5) can all increase membrane fluidity (Goldstein, 1984; Rozental et al., 2001), a property that probably explains their ability to also close gap-junctions in some systems (Rozental et al., 2001). However, except for oleamide, these agents did not in themselves affect J_{dw} ; and the oleamide-evoked J_{dw} inhibition was not large. This observation raises the possibility that any putative increase in water movement across the more disordered bilayer was offset by closure of a water-transporting channel within the membrane, thereby precluding a subsequent IBMX-effect on the channel. Clearly, biochemical and molecular studies are needed to unravel the processes underlying these data.

In brief, our studies tested the hypothesis that cAMP-elevation might enhance P_{dw} across the conjunctiva, an idea that remains open should PKA activity up-regulate AQP5 transcription and translation as found in lung cells (Sidhaye et al., 2005; Yang et al., 2003). Yet, the short-term effects (within 15 min) of drugs expected to increase intracellular cAMP levels were to elicit an inhibition of P_{dw} . This response was particularly evident with PDE inhibitors, which evoke instantaneous, PKA-dependent increases in apical Cl^- and Na^+ , and basolateral K^+ permeabilities (as measured by the I_{sc} ; Turner et al., 2000), while saliently reducing a transepithelial water flow that is transcellular, with the latter occurring in an H89-insensitive manner due to the closure of channels that seem to be located in the basolateral domain.

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Figure 1.

Transepithelial water fluxes were measured across conjunctival epithelia at 3 temperatures (286°K, 299°K, and 312°K) under control conditions (closed circles) and repeated at the same temperatures with 0.1 mM IBMX present in the apical bath (open circles). Points are means $\pm$ SEM's of $\ln P_{dw}$ with $n=4$ conjunctivae. The increase in slope (from 5.35 to 6.97 kcal per mole) elicited by the presence of IBMX was significant as paired data, $P<0.02$.

Table 1

Effects of cAMP-elevating agents on unidirectional water fluxes, short-circuit current, and transepithelial resistance of the rabbit conjunctival epithelium.

		J_{wv} , $\mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$			I_{sc} , $\mu\text{A}\cdot\text{cm}^{-2}$			R_p , $\text{k}\Omega\cdot\text{cm}^2$		
		Control	Db-cAMP (1 mM)	IBMX (100 μM)	Control	Db-cAMP (1 mM)	IBMX (100 μM)	Control	Db-cAMP (1 mM)	IBMX (100 μM)
Protocol A	Mean:	7.56	6.92	5.96*	20.9	28.2 [†]	37.8 [†]	0.95	0.79*	0.55*
	n:	8	8	8	8	8	8	8	8	8
	SE:	1.16	0.98	1.01	3.3	4.9	5.8	0.12	0.09	0.04
		Control	IBMX (100 μM)	FSK (10 μM)	Control	IBMX (100 μM)	FSK (10 μM)	Control	IBMX (100 μM)	FSK (10 μM)
Protocol B	Mean:	7.01	5.14*	4.86	17.1	29.3 [†]	20.7	0.46	0.38*	0.38
	n:	10	10	10	10	10	10	10	10	10
	SE:	1.51	1.04	1.10	2.0	2.6	1.9	0.07	0.04	0.04
		Control	FSK (10 μM)	IBMX (100 μM)	Control	FSK (10 μM)	IBMX (100 μM)	Control	FSK (10 μM)	IBMX (100 μM)
Protocol C	Mean:	6.22	5.96*	4.49*	15.4	30.6 [†]	22.7	0.51	0.40*	0.39
	n:	10	10	10	10	10	10	10	10	10
	SE:	0.80	0.81	0.50	3.2	4.2	2.9	0.10	0.06	0.06
		Control	Rolipram (100 μM)	IBMX (100 μM)	Control	Rolipram (100 μM)	IBMX (100 μM)	Control	Rolipram (100 μM)	IBMX (100 μM)
Protocol D	Mean:	7.91	5.56*	4.89*	17.2	25.6 [†]	20.6*	0.58	0.45*	0.42
	n:	8	8	8	8	8	8	8	8	8
	SE:	1.21	0.87	0.83	4.0	5.1	4.6	0.17	0.09	0.08
		Control	IBMX (100 μM)	Rolipram (100 μM)	Control	IBMX (100 μM)	Rolipram (100 μM)	Control	IBMX (100 μM)	Rolipram (100 μM)
Protocol E	Mean:	10.32	7.31*	7.07	15.6	30.5 [†]	22.4	0.56	0.43*	0.43
	n:	4	4	4	4	4	4	4	4	4
	SE:	0.68	0.64	0.32	6.3	9.1	6.0	0.14	0.07	0.06

J_{wv} , unidirectional water fluxes; I_{sc} , short-circuit current; R_t , transepithelial resistance. Water fluxes were measured in the tear-to-stromal direction; after 1h of control sampling (at 15-min intervals), the first test compound was added to the apical, radiolabeled bath, followed by the second compound 1h later. Sampling continued uninterrupted in the presence of the drugs for a total duration of the experiments of 3 h.

* Significantly less than respective antecedent ($P < 0.05$), as paired data.

[†] Significantly larger than respective antecedent ($P < 0.05$), as paired data.

Effects of the PKA inhibitor, H89, and IBMX on unidirectional water fluxes and short-circuit current across the rabbit conjunctival epithelium.

		J_{dw} , $\mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$		I_{sc} , $\mu\text{A}\cdot\text{cm}^{-2}$	
Protocol A		Control	H89 (10 μM)	Control	H89 (10 μM)
Mean:	6.81			21.6	
n:	4			4	
SE:	1.02			4.4	
		2 Hours H89-pretreatment (10 μM)		Control	
Mean:	-			16.9	
n:	-			3	
SE:	-			0.9	
		2 Hours H89-pretreatment (10 μM)		Control	
Mean:	-			16.9	
n:	-			3	
SE:	-			0.9	

J_{dw} , unidirectional water fluxes; I_{sc} , short-circuit current. In protocol A, water fluxes were measured in the tear-to-stromal direction; after 1h of control sampling (at 15-min intervals), H89 was added to the apical, radiolabeled bath, followed by IBMX 1h later. Sampling continued uninterrupted in the presence of the drugs for a total duration of the experiments of 3 h. In protocol B, the control electrical parameters of freshly mounted preparations were recorded, followed by the introduction of H89 to the apical bath for a 2-h pretreatment period before the addition of tritiated water to initiate the sampling periods. After the drawing of 4 baseline samples over the course of an hour from the stromal-side bath to represent the flux across the H89-pretreated epithelium, IBMX was added to the apical bath for an additional hour of sampling.

* Significantly less than respective antecedent ($P < 0.02$), as paired data.

Table 3
Effects of the cytoskeletal disruptors, cytochalasin B and colchicine, and of IBMX on unidirectional water fluxes and short-circuit current across the rabbit conjunctival epithelium.

		J_{dw} , $\mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$		I_{sc} , $\mu\text{A}\cdot\text{cm}^{-2}$		
Protocol A		Control	Cytochalasin B (30 μM)	IBMX (100 μM)	Cytochalasin B (30 μM)	IBMX (100 μM)
Mean:	8.67	6.91*	17.0*	25.3	17.0*	9.5*
n:	6	6	6	6	6	6
SE:	0.8	0.67	2.7	4.7	2.7	1.6
Protocol B		Control	Colchicine (30 μM)	IBMX (100 μM)	Colchicine (30 μM)	IBMX (100 μM)
Mean:	5.96	5.56*	22.4	19.8	22.4	29.8 [†]
n:	3	3	3	3	3	3
SE:	0.26	0.28	4.5	3.8	4.5	3.8

J_{dw} , unidirectional water fluxes; I_{sc} , short-circuit current. Water fluxes were measured in the tear-to-stromal direction; after 1h of control sampling (at 15-min intervals), either cytochalasin B, or colchicine, was added to the apical, radiolabeled bath, followed by IBMX 1h later. Sampling continued uninterrupted in the presence of the drugs for a total duration of the experiments of 3 h.

* Significantly less than respective antecedent ($P < 0.025$), as paired data.

† Significantly larger than respective antecedent ($P < 0.01$), as paired data.

Table 4
Effects of various channel blockers and IBMX on unidirectional water fluxes and short-circuit current across the rabbit conjunctival epithelium.

	J_{dw} , $\mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$		I_{sc} , $\mu\text{A}\cdot\text{cm}^{-2}$	
Protocol A	Control		Control	
Mean:	7.51	IBMX (100 μM) 5.50*	Control	IBMX (100 μM) 6.0
n:	4	Cu ²⁺ (1 mM) 6.81*	14.5	6.5*
SE:	1.06	4	4	4
		0.98	2.7	1.4
Protocol B	Control		Control	
Mean:	4.58	Glibenclamide (300 μM) 4.08*	Control	IBMX (100 μM) 9.5†
n:	5	5	15.5	5
SE:	0.29	0.24	5	5
			0.3	0.5
Protocol C	Control		Control	
Mean:	8.89	NPPB (100 μM) 8.05*	Control	IBMX (100 μM) 3.9
n:	3	3	11.5	3
SE:	1.12	1.00	3	3
			3.4	0.8
Protocol D	Control		Control	
Mean:	8.52	Niflumic Acid (100 μM) 8.41	Control	IBMX (100 μM) 13.6†
n:	6	6	14.2	6
SE:	0.95	0.99	6	6
			1.9	1.7
Protocol E	Control		Control	
Mean:	8.00	Ba ²⁺ (10 mM) 7.08*	Control	IBMX (100 μM) 3.1
n:	5	5	15.4	5
SE:	0.88	1.08	5	5
			1.9	1.2

J_{dw} , unidirectional water fluxes; I_{sc} , short-circuit current. Water fluxes were measured in the tear-to-stromal direction under conditions identical to those described in the Table 1 legend. Most agents were added to the apical bath, except Cu²⁺ and Ba²⁺, which were added bilaterally, with their concentrations maintained in the replacement solutions.

* Significantly less than respective antecedent ($P < 0.05$), as paired data.

† Significantly larger than respective antecedent ($P < 0.05$), as paired data.

Table 5

Effects of putative uncoupling agents and IBMX on unidirectional water fluxes and short-circuit current across the rabbit conjunctival epithelium.

	$J_{wv}, \mu\text{l}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$		$I_{sc}, \mu\text{A}\cdot\text{cm}^{-2}$	
Protocol A	Control	1-Octanol (2 mM)	Control	Octanol (2 mM)
Mean:	6.99	6.87	21.8	6.48*
n:	7	7	7	7
SE:	0.60	0.57	2.4	0.8
Protocol B	Control	1-Heptanol (5 mM)	Control	Heptanol (5 mM)
Mean:	6.46	6.42	16.2	8.4*
n:	5	5	5	5
SE:	1.71	1.49	2.2	1.0
Protocol C	Control	1-Heptanol (10 mM)	Control	Heptanol (10 mM)
Mean:	8.96	8.67	13.3	-0.1*
n:	5	5	5	5
SE:	0.86	1.14	1.1	1.8
Protocol D	Control	Oleamide (1 mM)	Control	Oleamide (1 mM)
Mean:	8.45	7.86*	14.1	18.6†
n:	6	6	6	6
SE:	1.07	1.08	1.2	1.8
Protocol E	Control	Arachidonate (200 μM)	Control	Arachidonate (200 μM)
Mean:	10.77	10.60	12.3	20.9†
n:	5	5	5	5
SE:	3.15	3.40	1.9	2.4
				IBMx (100 μM)
				9.0
				5
				1.9
				30.2†
				6
				2.6
				23.1†
				5
				3.0

J_{wv} , unidirectional water fluxes; I_{sc} , short-circuit current. Water fluxes were measured in the tear-to-stromal direction under conditions identical to those described in the Table 1 legend.

* Significantly less than respective antecedent ($P < 0.05$), as paired data.

† Significantly larger than respective antecedent ($P < 0.05$), as paired data.